Detection of highly and minimally leukotoxic *Actinobacillus actinomycetemcomitans* strains in patients with periodontal disease

Detecção de cepas de Actinobacillus actinomycetemcomitans *de máxima e mínima leucotoxicidade em pacientes com doença periodontal*

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ABSTRACT: This study examined the prevalence of highly and minimally leukotoxic *Actinobacillus actinomycetemcomitans* in patients with periodontal disease. Pooled subgingival plaque samples from 136 patients with some form of periodontal disease were examined. Subjects were between 14 and 76 years of age. Clinical examinations included periodontal pocket depth (PD), plaque index (PI) and bleeding index (BI). The obtained plaque samples were examined for the presence of highly or minimally leukotoxic *A. actinomycetemcomitans* strains by the polymerase chain reaction (PCR). Chi-square and logistic regression were performed to evaluate the results. Forty-seven subjects were diagnosed with gingivitis, 70 with chronic periodontitis and 19 with aggressive periodontitis. According to chi-square there was no significant correlation detected between PD ($\chi^2 = 0.73$), PI ($\chi^2 = 0.35$), BI ($\chi^2 = 0.09$) and the presence of the highly leukotoxic *A. actinomycetemcomitans.* The highly leukotoxic *A. actinomycetemcomitans* strains were correlated with subjects that were 28 years of age and younger ($\chi^2 = 7.41$). There was a significant correlation between highly leukotoxic *A. actinomycetemcomitans* and aggressive periodontitis ($\chi^2 = 22.06$). This study of a Brazilian cohort confirms the strong association between highly leukotoxic *A. actinomycetemcomitans* strains and the presence of aggressive periodontitis.

DESCRIPTORS: *Actinobacillus actinomycetemcomitans*; Leukotoxin; Gingivitis; Periodontitis; Polymerase chain reaction.

RESUMO: O presente estudo avaliou a prevalência de *Actinobacillus actinomycetemcomitans* de máxima e mínima leucotoxicidade em indivíduos com doença periodontal. Foram analisadas amostras de placa bacteriana subgengival de 136 indivíduos, entre 14 e 76 anos de idade, com algum tipo de doença periodontal. Os parâmetros clínicos avaliados incluíram profundidade de sondagem (PS), índice de placa bacteriana (IPB) e índice de sangramento gengival (ISG). A presença de amostras de *A. actinomycetemcomitans* de máxima e mínima leucotoxicidade foi avaliada por reação em cadeia da polimerase (PCR). Os resultados foram analisados empregando-se teste qui-quadrado e análise de regressão logística. Quarenta e sete indivíduos apresentaram gengivite, 70 indivíduos periodontite crônica e 19 indivíduos periodontite agressiva. O teste qui-quadrado não demonstrou correlação significativa entre PS ($\chi^2 = 0,73$), IPB ($\chi^2 = 0,35$), ISG ($\chi^2 = 0,09$) e a presença de amostras de máxima leucotoxicidade. *A. actinomycetemcomitans* de máxima leucotoxicidade apresentou associação com indivíduos com idade inferior a 28 anos ($\chi^2 = 22,06$). Os resultados observados nessa população brasileira confirmam a forte associação existente entre amostras de *A. actinomycetemcomitans* de máxima leucotoxicidade e periodontite agressiva.

DESCRITORES: *Actinobacillus actinomycetemcomitans*; Leucotoxina; Gengivite; Periodontite; Reação em cadeia da polimerase.

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INTRODUCTION

The presence of periodontal pathogens can be frequently detected in healthy subjects. This indicates that not all humans are equally susceptible and/or that there is variation in virulence and pathogenic potential (Tonetti e Mombelli[23], 1999; Zadeh *et al.*[26], 1999). Bacteria associated with periodontal disease have a number of virulence factors such as endo- and exotoxins, that can directly damage host tissues, and immune evasion strategies which shield the bacteria from host defenses.

Every strain of *A. actinomycetemcomitans* studied to date is resistant to serum-mediated killing. They are not killed by unseparated serum and any serum component by implication, which would include the membrane attack complex, platelet-derived β-lysin, acute phase proteins and lysozyme. To clear an infection by a serum-resistant organism necessarily involves host phagocytes (Zadeh *et al.*[26], 1999). However, *A. actinomycetemcomitans* has been shown to be capable of inhibiting polymorphonuclear leukocytes chemotaxis (Van Dyke *et al.*[24], 1982; Fives-Taylor *et al.*[7], 1996). Furthermore, phagocytosis inhibition is associated with *A. actinomycetemcomitans* Fc binding proteins. *A. actinomycetemcomitans* can also inhibit the leukocytes fusion with lysosomes (Fives-Taylor *et al.*[8], 1999).

A. actinomycetemcomitans produce a leukotoxin (ltx) that is an RTX (repeats in toxin) toxin. The RTX family members share similar gene organization and are expressed from an operon consisting of four genes that are designated C (ltxC), A (ltxA), B (ltxB) and D (ltxD) in transcriptional order. The structural gene is designated as the gene A and the three remaining genes flanking the A gene are required to activate and transport the toxin (Lally *et al.*[12], 1989). Leukotoxins are cell-specific. The toxin binds to neutrophils, monocytes and a subset of lymphocytes (Zadeh *et al.*[26], 1999). Cell lysis might be induced by the rapid formation of highly conductive ion channels that lead to membrane depolarization, loss of intracellular K^+, osmotic swelling, and subsequently cell death (Slots[20], 1994; Lally *et al.*[12], 1996; Karakelian *et al.*[11], 1998; Fives-Taylor *et al.*[8], 1999). The expression of leukotoxins is tightly regulated in many strains, although some strains produce high levels of leukotoxins (Brogan *et al.*[5], 1994; Mombelli *et al.*[15], 1999; Paju *et al.*[17], 2000). The promoter re-gions of high producers have a 530 bp deletion that may be involved in repressing leukotoxin expression (Brogan *et al.*[4], 1994). Leukotoxins may protect *A. actinomycetemcomitans* against phagocytosis by human polymorphonuclear leukocytes (Johansson *et al.*[10], 2000). Patients with aggressive periodontitis were shown to have a substantially higher prevalence of highly leukotoxic strains than chronic periodontitis individuals (Zambon *et al.*[28], 1996; Haraszthy *et al.*[9], 2000).

The aim of the present study was to examine the prevalence of highly and minimally leukotoxic *Actinobacillus actinomycetemcomitans* in patients with periodontal disease.

MATERIALS AND METHODS
Subject population

A total of 136 subjects, males and females, aged from 14 to 76 years with gingivitis (47), chronic (70) and aggressive (19) periodontitis were enrolled in this study. Individuals were previously classified as gingivitis, chronic or aggressive periodontitis patients, based upon clinical and radiographic findings (periodontal probing depth - PD, loss of clinical attachment and periapical radiographs) and on the diagnostic criteria presented in the World Workshop of Periodontology 1999 (AAP[2], 1999). Clinical examinations also included Plaque Index - PI and Bleeding Index - BI (Ainamo, Bay[1], 1975).

Subjects were excluded if any of the following conditions were detected: use of systemic antibiotic in the previous 6 months and periodontal therapy undertaken less than 12 months before the study. All subject participation was preceded by approval of all protocols by the appropriate institutional review board and the obtainment of a signed consent from participants.

Microbial Sampling and Polymerase Chain Reaction

Pooled subgingival plaque samples were taken from five periodontal sites (> PD). After the removal of supragingival plaque, subgingival plaque samples were taken with sterile paper points (Tanari, Tanariman Industrial Ltda., Manacapuru, Brazil) inserted to the depth of the pockets or sulci from the mesial aspect of five different teeth from each subject. The paper points were removed after 15 seconds, and immediately placed in phosphate-buffered saline (pH 7.4) on ice. The bacterial cells were pelleted at 12,000 x g for 1 minute in a

microfuge (Eppendorf) and DNA was isolated from the samples (Instagene Purification Matrix, Bio Rad, Hercules, USA).

PCR was performed using a thermocycler (Perkin Elmer Cetus) with *Taq* polymerase (Amershan, Arlington Heights, USA). Using previously published sequences (Brogan *et al.*[5], 1994), 2 oligonucleotide primers were used to the *A. actinomycetemcomitans* leukotoxin promoter region:

• A - 5'-GCAGGATCCATATTAAATCTCCTTGT-3';
• B - 5'-GCGGTCGACAACCTGATAACAGTATT-3'.

They were used to amplify either a 492 base pair product characteristic of highly leukotoxic *A. actinomycetemcomitans* or a 1022 base pair product characteristic of minimally leukotoxic *A. actinomycetemcomitans*. PCR was performed using standard conditions. The reaction mixture consisted of 100 µl of 10 mM Tris-HCl pH 8.3 (Amershan, Arlington Heights, USA), 50 mM KCl (Amershan, Arlington Heights, USA), 1.5 mM MgCl$_2$ (Amershan, Arlington Heights, USA), 100 µM each of dATP, dCTP, dGTP, dTTP (Amershan, Arlington Heights, USA), 0.001% (w/v) gelatin, 100 ng of each primer (Invitrogen/Gibco, San Diego, USA), 0.5 U *Taq* polymerase, and 100 ng of genomic DNA. The thermocycle profile was 25 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C. The amplification products were analyzed by electrophoresis in 2% submarine agarose gels (Sigma, Dorset, UK) and visualized by UV illumination (Bio Rad, Hercules, USA) following ethidium bromide (Amershan, Arlington Heights, USA) staining. These agarose gels were compared with both positive (JP2 and 652 strains) and negative controls.

Statistical analysis

Chi-square and logistic regression were performed to evaluate the results. The level of significance considered was set at p < 0.05. The relation between variants and periodontal diagnosis was established by logistic regression. Age, gender, smoking, PD, PI, BI, PCR detection and highly or minimally leukotoxic *A. actinomycetemcomitans* were considered in separated or multiple analyses. The probability of aggressive periodontitis – P(AP) – was calculated as follows:

$$P(AP) = \frac{1}{1 + \exp\,(5.58 - 0.42\ \text{age} + 0.74\ \text{periodontal pocket depth})}$$
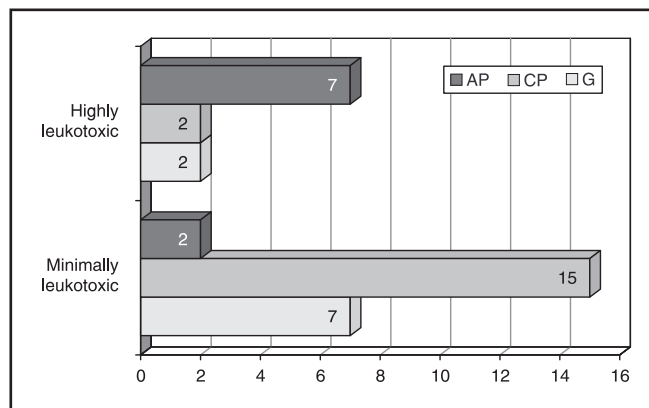
GRAPH 1 - Highly and minimally leukotoxic *Actinobacillus actinomycetemcomitans* according to periodontal *status*. G - gingivitis (n = 47); CP - chronic periodontitis (n = 70); AP - aggressive periodontitis (n = 19).

RESULTS

Graph 1 shows the distribution of highly and minimally leukotoxic *A. actinomycetemcomitans*. The major prevalence of highly leukotoxic *A. actinomycetemcomitans* was observed in aggressive periodontitis subjects followed by chronic periodontitis and gingivitis (χ^2 = 22.06). No statistically significant difference was observed between gingivitis and chronic periodontitis related to highly leukotoxic *A. actinomycetemcomitans* (χ^2 = 0.17). In contrast, highly leukotoxic *A. actinomycetemcomitans* was correlated with subjects 28 years of age and younger (χ^2 = 7.41).

Minimally leukotoxic *A. actinomycetemcomitans* showed no correlation with periodontal *status* (χ^2 = 0.91). No association between PD (χ^2 = 0.73), PI (χ^2 = 0.35) and BI (χ^2 = 0.09) and highly and minimally leukotoxic *A. actinomycetemcomitans* was detected.

Separated logistic regression analyses showed correlation between periodontal *status* and age, highly leukotoxic *A. actinomycetemcomitans* and PI (Table 1). Multiple logistic regression analyses showed correlation between periodontal disease, age and PD (Table 2).

The probability of aggressive periodontitis was calculated. Graph 2 shows the results. The minimum and maximum values of PD found among all subjects included in the present study were applied in multiple logistic regression. Age was also considered for analyses.

TABLE 1 - Estimate coefficient, p value and statistically significance established by logistic regression related to isolated analyses of each considered variant.

Variants	Coefficient	p value	Results
Age	–0.33	0.00	< age > probability of AP
Highly leukotoxic *A. actinomycetemcomitans*	2.99	0.00	Presence > probability of AP
Plaque index	–0.30	0.05	No plaque > probability of AP
Bleeding index	–0.90	0.10	ns
Smoking	–0.62	0.28	ns
Minimally leukotoxic *A. actinomycetemcomitans*	0.73	0.29	ns
PCR detection	0.47	0.32	ns
Periodontal pocket depth	–0.11	0.55	ns
Gender	0.06	0.92	ns

AP - aggressive periodontitis; PCR detection - presence of *A. actinomycetemcomitans*; PCR - polymerase chain reaction; ns - not significant.

TABLE 2 - Results of multiple logistic analyses.

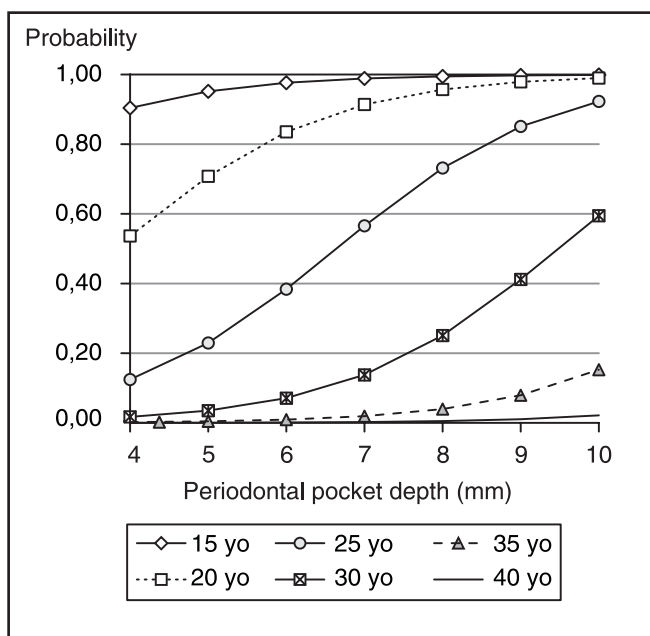| Variant	Age	Periodontal pocket depth
Coefficient	–0.42	0.74
p value	0.00	0.05

DISCUSSION

A. actinomycetemcomitans is an important pathogen in some forms of periodontitis. Aggressive periodontitis is the most notorious disease associated with *A. actinomycetemcomitans.* The prevalence of this pathogen is nearly 90% in aggressive periodontitis and 30-50% in severe chronic periodontitis. Furthermore, one of the most important virulence factors of *A. actinomycetemcomitans* is leukotoxin (Lally *et al.*[12], 1996; Karakelian *et al.*[11], 1998; Fives-Taylor *et al.*[8], 1999; Zadeh *et al.*[26], 1999). In the present study, the periodontal *status* was associated with highly leukotoxic *A. actinomycetemcomitans.* There was significant correlation between highly leukotoxic strain and aggressive periodontitis (χ^2 = 22.06) (Graph 1). Additionally, we observed that the highly leukotoxic *A. actinomycetemcomitans* strain was correlated with subjects 28 years of age and younger (χ^2 = 7.41). These data agree with the results obtained by Ávila-Campos, Velásquez-Meléndez[3] (2002) who observed that *A. actinomycetemcomitans* was associated with disease in fifty Brazilian patients with clinical and radiographic evidence of alveolar bone loss confined to the molar and incisor teeth. Nakagawa *et al.*[16], 2001, analyzed the serumal and salivary levels of antibodies against the leukotoxin produced by *A. actinomycetemcomitans* in Brazilian patients with aggressive periodontitis and in healthy controls. The authors found significantly higher serumal levels of IgG in patients with aggressive periodontitis, when they were compared with the healthy controls. However, Lima *et al.*[13], 2001, isolated a high leukotoxin producer from a healthy subject and moderate producers from periodontal disease patients.

On the other hand, no relation was observed between plaque index (χ^2 = 0.35), bleeding index (χ^2 = 0.09) and highly and minimally leukotoxic *A. actinomycetemcomitans*. These data are in accordance with other studies which strongly showed that there is more probability to detect *A. actinomycetemcomitans* when clinical signs of inflammation were evident (Mombelli *et al.*[14], 1995; Zambon *et al.*[27], 1994; Tanner *et al.*[22], 1996). Particularly in chronic periodontitis subjects, *A. actinomycetemcomitans* was weakly related to the bleeding index, while there was no correlation between the organism and any clinical index in aggressive periodontitis subjects (Bonta *et al.*[4] 1985; Renvert *et al.*[18] 1997; Shiloah e Patters[19], 1997).

When periodontal pocket depth is considered, different authors have related *A. actinomycetemcomitans* to deeper pockets (Slots *et al.*[21], 1980; Wolff *et al.*[25], 1993; Zambon[27], 1994; Tanner *et al.*[22], 1996). In the present study no correlation was observed between periodontal pocket depth and highly and minimally leukotoxic *A. actinomycetemcomitans*. However, multiple logistic regression analyses showed that periodontal pocket depth was related to highly leukotoxic *A. actinomycetemcomitans* (p < 0.05).

Separated logistic regression was applied to analyze the relation between risk factors and periodontal *status* (Table 1). Age and PD were strongly associated with aggressive periodontitis (p < 0.05) according to multiple logistic regression (Table 2).

GRAPH 2 - Probability of aggressive periodontitis according to age and periodontal pocket depth (mm) of the studied population; yo: years old.

Different ages and values of PD found in the present study originated a diagram of probabilities for aggressive periodontitis. Subjects 20 years of age or younger and PD between 9 and 10 mm showed 100% of probability of aggressive periodontitis. On the other hand, subjects 40 years of age or older, even showing similar PD (9 to 10 mm) had probability close to zero to exhibit aggressive periodontitis (Graph 2). The present research showed an association between age, PD and aggressive periodontitis.

A. actinomycetemcomitans is implicated in aggressive periodontitis and it is generally considered a major etiologic microorganism in this form of disease. However, the role of the pathogen in chronic periodontitis is more difficult to make clear since *A. actinomycetemcomitans* is found in a much more microbiologically complex and diverse plaque ecology compared to *A. actinomycetemcomitans* infections in aggressive periodontitis (Zambon[27], 1994). The relationships between bacteria in chronic periodontitis are frequent. For example, *P. gingivalis* and *P. intermedia* produce proteases that can decrease the toxicity of *A. actinomycetemcomitans.* Thus, in some patients *A. actinomycetemcomitans* can exhibit a complementary role in developing periodontal disease (Chen, Slots[6], 1999).

CONCLUSIONS

The major prevalence of highly leukotoxic *A. actinomycetemcomitans* was observed in aggressive periodontitis. Minimally leukotoxic strains showed no correlation with periodontal *status*. This study of a Brazilian cohort confirms the strong association between highly leukotoxic *A. actinomycetemcomitans* strains and the presence of aggressive periodontitis.

ACKNOWLEDGEMENTS

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